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Effectiveness of Different Molecular Weights and Concentrations of Chitosan on Enteric Viral Surrogates

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To the Graduate Council:

I am submitting herewith a thesis written by Robert Hamilton Davis entitled "Effectiveness of Different Molecular Weights and Concentrations of Chitosan on Enteric Viral Surrogates." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Svetlana Zivanovic, Major Professor

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Effectiveness of Different Molecular Weights and Concentrations of Chitosan on Enteric Viral Surrogates

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DEDICATION

This thesis is dedicated to the loving memory of my mother

Mrs. Helen H. Davis
April 20,1960 – June 11, 2006

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ABSTRACT

Chitosan is known to be antibacterial and antifungal, but information on its effectiveness against foodborne viruses is limited. Enteric viruses are a major concern in food safety, especially human noroviruses which are the leading cause of nonbacterial gastroenteritis. The overall goal of this research was to determine the antiviral effectiveness of chitosan. The specific objectives were to determine the effects of molecular weight (MW) and concentration of chitosan against the cultivable enteric viral surrogates, feline calicivirus (FCV-F9), murine norovirus (MNV-1), and bacteriophages (MS2 and phiX174). Purified chitosans (53, 222, 307, 421, ~1,150kDa) were dissolved in water, 1% acetic acid, or aqueous HCl (pH= 4.3), and sterilized by membrane filtration. The solutions were mixed with equal volume of virus suspension to obtain a virus titer of 5 log PFU/ml and chitosan concentration of 0.7% for all five MW and 0.7, 1.0, 1.25, and 1.5% for 53 and 222kDa. The samples were incubated for 3 hr at 37°C before viral enumeration. Controls included untreated viruses in PBS, in PBS with acetic acid, and in PBS with HCl. Chitosan showed the greatest reduction of MS2, followed by FCV-F9, phi X174, and MNV-1. A MW effect was seen with MS2, with higher MW being more efficient, and 0.7% of ~1,150kDa causing complete inactivation. Increasing the concentration of chitosan from 0.7 to 1.5% reduced the titer of MS2 and FCV-F9 by 5.16 and 2.91 logs, respectively. Although chitosan was ineffective against MNV-1, its ability to significantly reduce MS2 and FCV-F9, suggest its use for future foodborne viral control.

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CHAPTER I

INTRODUCTION

Foodborne disease outbreaks, especially those caused by viruses, have become a major concern in the food industry. Chitosan has shown promise as a potential antimicrobial (Devlieghere et al., 2004), food additive, such as a clarifying agent or antioxidant, (Shahidi et al., 1999), and packaging material (Shahidi et al., 1999) and has been approved by EPA as a biopesticide (Docket No. EPA-HQ-OPP-2007-0566-0019). Therefore, this study was done to determine the effectiveness of chitosan against surrogates of enteric viruses commonly associated with foodborne outbreaks.

The overall goal of this research was to determine antiviral efficiency of chitosan. The specific objectives were:

1. To determine the effect of chitosan against the enteric virus surrogates phi X174, MS2, feline calicivirus (FCV-F9), and murine norovirus (MNV-1).
2. To determine the effect of molecular weight (MW) of chitosan, ranging from 53 to ~1,150 kDa.
3. To determine the effect of concentration of chitosan (0.7- 1.5%) on the enteric virus surrogates

1.2 Hypotheses

The hypotheses for these objectives were as follows:

1. Increasing the molecular weight of chitosan would increase the antiviral activity because the longer chains would able to wrap around and damage the viral structure more than the lower molecular weight.
2. Increasing the concentration would improve the antiviral activity of chitosan due to more positive charges being able to interact with negatively charged components of the viral capsid.

1.3 Explanation for molecular weights and concentrations chosen

The molecular weights of chitosan chosen for this study covered the majority of the chitosan molecular weight spectrum. Molecular weight chitosan lower than 50 kDa was not used because the study by Su et al. (2009) already determined that they were not effective against these surrogates.Similarly, the concentrations of 0.7% and above were selected because the effect of lower concentration was already reported in literature (Su et al., 2009). However, the increase in chitosan concentration was limited to 1.5% due to the increase in the viscosity.

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CHAPTER II

LITERATURE REVIEW

1.1. Chitosan

Chitosan, a linear cationic polysaccharide, is a deacteylated derivative of chitin, which is the second most abundant polysaccharide in nature. Chitin is the major structural biopolymer in crustacean shells, fungi, and insects (Roller et al. 1999) and chitosan is naturally present in some fungi, such as zygomycetes (Shahidi et al., 1999). Chitosan is composed of at least 70% glucosamine and no more than 30% acetyl-glucosamine units bound by β (1-4) glycosidic bonds. It is typically described by its molecular weight (MW), which ranges between 50-1000 kDa, and degree deacetylation (DDA), which ranges between 70-100%. Although chitosan is approved as a food additive in food in Japan and Korea (No et al., 2007), it is not generally recognized as safe (GRAS) in the United States. However, it has been accepted as a bio pesticide (Docket No. EPA-HQ-OPP-2007-0566-0019), sold as a dietary supplement, and allowed as an additive to dog food (Park et al., 2002). Chitosan is used to form gels, films, beads, and fibers (Guibal 2004; Sankararamakrishnan et al., 2006; Zivanovic et al. 2008), in waste water management as a chelator (Shahidi et al., 1999), and in medicine as wound dressings (Koide, 1998). Chitosan has been researched for use in the food industry in various fields, including as an antimicrobial, antioxidant, thickening agent in beverages, clarifying agent in juice, and as a packaging material (Devlieghere et al., 2004; Shahidi et al., 1999; Xie et al., 2001).

Since a major source of chitosan is shellfish waste and shellfish is one of the most common allergens, there is a concern that chitosan might act as an allergen as well. However, Gray et al. (2004) determined that it was safe for people with shellfish allergies to use glucosamine supplements because it is the proteins of the flesh of the fish, and not the shell, that cause the reaction. Furthermore, the extraction and preparation of chitosan should remove all proteins including those that may be potential allergens (Muzzarelli 2010). Due to the aggressive chemical processes used to extract chitin and produce chitosan, the product should not be considered as part of the living organism that biosynthesized it, just as the maize starch is not considered part of maize plant after the wet milling (Muzzarelli, 2010).

1.2. Mechanisms of antibacterial action of chitosan

Antibacterial properties of chitosan have been widely investigated and confirmed (Coma et al., 2003; Dutta et al., 2009; Kong et al., 2008; Liu et al., 2004; Zivanovic et al., 2004). However, there are several mechanisms that have been proposed to explain the antimicrobial mechanisms of chitosan. The first proposed antibacterial mechanism of chitosan occurs due to electrostatic interactions between the chitosan amine group and the negatively charged components (lipopolysaccharides and proteins) of the outer cell causing distortion of the cellular membrane and leakage of intracellular material (Coma et al., 2003; Dutta et al., 2009; Helander et al., 2001; Kong et al., 2008; Liu et al., 2004). A study by Liu et al. (2004) treated *S. aureus* and *E. coli* with 0.5 and 0.25% 78 kDa chitosan causing a 1 log reduction after 5 min, complete inactivation of *E. coli* after 120

min, and no change in *S. aureus* after 120 min. The cells were examined by transmission electron microscope and *E. coli* was found to have an altered and chitosan-covered outer membrane, but no damage to the inner membrane. On the other hand, *S. aureus* showed leakage of intracellular material and new cells were found to form without membranes or cell walls on the outside. Another study by Helander et al. (2001), treated *E. coli* and *S.* Typhimurium with 0.025% of 85% DDA chitosan, which altered the outer membrane of the cell and formed a layer around *E. coli*.

Zheng and Zhu (2003) suggested that chitosan interrupts the physiological activities of the cell, but affects Gram positive and Gram negative cells differently. They proposed that higher molecular weight chitosan forms a polymer membrane to prevent nutrients from leaving and entering the cell on Gram positive organisms, while lower molecular weight chitosan enters the Gram negative cell binding to electronegative substances, which causes flocculation in the cytoplasm and disruption of physiological processes (Zheng and Zhu, 2003).

The third proposed mechanism of action is the inhibition of mRNA and protein synthesis by penetrating the cell of the microorganism and binding with DNA (Sudarshan et al., 1992). Still another proposed antibacterial mechanism of chitosan is the chelation of essential nutrients needed for growth (Dutta et al., 2009). Kong et al. (2008) found that chitosan microspheres of 1456 kDa were chelating Mg^{2+} of the *E. coli* outer membrane causing destabilization of the cell. Still, the antibacterial property of chitosan could be a combination of all the proposed mechanisms of action depending on the type of microorganism and characteristics of chitosan.

1.3. Mechanisms of antifungal properties of chitosan

Similar mechanisms have been proposed for antifungal activity of chitosan. The first mechanism involves the interaction of chitosan with the cell plasma membrane causing leakage of intracellular material (El Ghaouth et al., 1992; Liu et al., 2007). Badaway et al. (2004) used chitosan and chitosan derivatives of less than 120 kDa (85% DDA) at 1% concentrations to find that *Botrytis cinerea* growth was reduced most likely due to chitosan causing a permeability change in the plasma membrane. Seyfarth et al. (2008) found that 120 kDa chitosan hydrochloride against *Candida albicans*, *C. krusei*, and *C. glabrata* caused disruption of the plasma membrane leading to permeable cells. Guerrero et al. (2005) found 0.1% chitosan to inhibit the growth and cause about a 1 log reduction in the first few min of treatment by causing structural defects in the cell wall of *Saccharomyces cerevisiae*. One study found chitosan to be effective at controlling *B. cinerea* infection in strawberries by either inducing natural plant resistant mechanisms or able to control its growth through fungistatic properties (Reddy et al., 2000). A second proposed mechanism involves the accumulation of chitosan in the cell wall to inhibit growth (El Ghaouth et al., 1992). El Ghaouth et al. (1992) found that chitosan caused leakage of amino acids as well as morphological changes due to the accumulation of chitosan in the cell wall of *Rhizopus stolinfer* and *B. cinerea*. The third proposed mechanism involves the chelation of Ca^{2+}, Zn^{2+}, and other essential minerals needed for growth (Cuero et al., 1991; Roller et al., 1999). A study by Roller et al. (1999) found that chitosan reduced the growth rates of *Mucor racemosus* by making Ca^{2+} and other essential minerals unavailable. The last proposed mechanism involves chitosan

interfering with conformation or physical properties of DNA in *Fusarium solani* (Hadwiger et al., 1981). Hadwiger et al. (1981) also found that 0.0002% of chemically cleaved chitosan applied to pea plants 24 hr in advance was able to protect against *F. solani*.

1.4. Antiviral properties of chitosan

The proposed antiviral mechanisms of chitosan are similar to the antifungal and antibacterial mechanisms, but vary depending on chitosan being applied pre-harvest or post-harvest. Most of the antiviral activity of chitosan has been studied on plants at pre-harvest. Chitosan has been shown to exhibit indirect antiviral activity at pre-harvest by penetrating the plant cell to induce resistance (Kulikov et al., 2006). On tobacco necrosis virus, 0.15% 76 kDa chitosan caused a 95.2% reduction by inducing abscisic acid production causing plant resistance to the virus (Iriti and Faoro, 2008).

The first proposed post-harvest mechanism is that chitosan causes structural damage to the virus (Kochkina et al., 2000a). Kochkina et al. (2000c) through electron microscopy have seen chitosan to cause loss in viral tail fibers with receptors and viral sheath contraction exposing DNA of phage T2 and 1-97A. Another mechanism proposes that chitosan interacts with the negative charge of the viral capsids (Kochkina et al., 2000b; Su et al., 2009). Su et al. 2009 found 0.7% 53 kDa chitosan caused a 1.7 log reduction of MS2, which could be due to the negatively charged MS2, which has an isoelectric point of 3.9 (Langlet et al., 2007), binding to the positively charged chitosan. The third proposed mechanism involves inhibiting phage infection (Kochkina et al.,

1995). A study found chitosan to inhibit the lytic infection bacteriophage of bacteriophage T2 and T7 after adsorption into the host by possibly attaching to the viral particles in the cell (Kochkina et al. 1995). Still another mechanism proposed chitosan interferes with a step in the replication process (Chirkov, 2002; Kochkina et al., 2000a). Kochkina et al. (2000a) found chitosan to be efficient at inhibiting phage replication of bacteriophage 1-97A in *B. thuringiensis*, which the authors thought to be due to the interruption of the intracellular reproduction of the phages. However, the effect of chitosan on foodborne viruses needs to be studied futher in order to gain a better understanding of effectiveness and mechanisms.

1.5. Effect of molecular weight on chitosans antimicrobial properties

Molecular weight (MW) is thought to play an important role in the antimicrobial effect of chitosan. Zheng and Zhu (2003) studied the effect of different molecular weights on Gram positive (*S. aureus*) and Gram negative (*E. coli*) bacteria. They found that as MW increased from less than 5 kDa to 305 kDa the antibacterial activity increased against Gram positive bacteria and decreased against Gram negative bacteria. The authors found the lower MW (<5 kDa at 0.25% concentration) inhibited gram negative *E. coli* by entering the cell and interrupting physiological activities of the cell, while the higher molecular weight (166 and 305 kDa at 0.25% concentration) inhibited Gram positive *S. aureus* by damaging the cell membrane. Devlieghere et al. (2004) determined that Gram negative bacteria were very susceptible to the effects of 43 kDa chitosan and that Gram positive bacteria varied greatly in response to the chitosan with some being inhibited,

while others were less affected. Zivanovic et al. (2004) found that 150 kDa chitosan inhibited Gram positive *Listeria monocytogenes* and Gram negative *Salmonella* Typhimurium better than ~5 kDa chitosan. Even No et al. (2002) found that chitosan between 28 – 1671 kDa inhibited the growth of both Gram negative (*E. coli, Pseudomonas fluorescens, Salmonella* Typhimurium, and *Vibrio parahaemolyticus*) and Gram positive (*Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus, Lactobacillus plantarum, Lactobacillus brevis*, and *Lactobacillus bulgaricus*) bacteria better than 1-22 kDa chitosan oligomers. Nevertheless, Gerasimenko et al. (2004) found that chitosans between 5 and 27 kDa were able to effectively inhibit Gram positive and Gram negative bacteria. Li et al. (2009) used 3, 50, and 1000 kDa chitosan to find that all three chitosans caused cellular membrane damage in *E. coli*, but 50 kDa caused the most.

Effect of molecular weight of chitosan against viruses has mostly been studied indirectly, *in vivo*.as chitosan increases plant resistance to viral attack. Chirkov et al. (1998) found that as the molecular weight of chitosan increased from 3, 8, to 50 kDa, the resistance of bean plants to bean mild mosaic virus increased. However, another study found the opposite trend for the same virus. This study used chitosan of 1.2, 2.2, 10.1, 30.3, and 40.4kDa to find that bean plants resistance to bean mild mosaic virus increased as MW decreased (Kulikov et al. 2006). A study by Su et al. (2009) has been done on the direct effects of chitosan of different molecular weights on human noroviruses surrogates, which showed an increasing antiviral activity trend. The authors found that 53 kDa chitosan reduced viral titers of bacteriophage MS2 and FCV-F9 by 1.70 and 4.21 log PFU/ml, respectively, while 5 kDa chitosan was only 0.98 and 1.41 log PFU/ml,

respectively. Su et al. (2009) also showed that chitosan was ineffective at inactivating MNV-1.

1.6. Effect of concentration on chitosans antimicrobial properties

Chitosan concentration is another factor thought to play an important role in determining the antimicrobial effect. In one study, chitosan of 78 kDa at 0.25 and 0.5% found that 0.5% chitosan showed more membrane damage of *S. aureus* and *E. coli* than 0.25% (Liu et al. 2004). Chitosan has been shown to disrupt outer membranes of *S.* Typhimurium at 0.01- 0.025%, but were unable to kill Gram negative bacteria until reaching 2% (Helander et al. 2001). One study found that antibacterial activity against *S.* Typhimurium, *E. coli*, and *Y. enterocolitica* increased as the concentration of chitosan increased from 0.5 to 2.5% (Wang, 1992). Kong et al. (2008) found an increase in antibacterial activity against *E. coli* as the concentration of 1456 kDa chitosan was increased from 0.02 to 0.1%. Zheng and Zhu (2003) found that as the concentration of <5 - 305 kDa chitosan increased from 0.25% to 1.0%, *E. coli* and *S. aureus* were reduced from little or no change to complete inactivation.

Chitosan concentration has also been shown to affect the antiviral activity. Kochkina et al. (1995) saw chitosan, chitosan acetate, and chitosan hydrochloride cause an increase from less than 50% to 100% infection inhibition against bacteriophage T2 and T7 as the concentration of chitosan increased from 0.000005 to 0.01%. Su et al. (2009) found an increase in concentration of both 53 kDa chitosan and 5 kDa chitosan from 0.175 to 0.7% to cause a statistically significant increase in the reduction of ~10^5 log PFU/ml FCV-F9

and ~10^7 log PFU/ml FCV-F9, while 5 kDa chitosan also caused a statistically significant increase in reduction of ~10^5 and ~10^7 log PFU/ml FCV-F9 and ~10^5 log PFU/ml MNV-1. However, for the same concentrations of both chitosans, the antiviral activity of chitosan either had no change or decreased against ~10^5 and ~10^7 log PFU/ml MS2 and ~10^7 log PFU/ml MNV-1 as chitosan concentration increased.

1.7. Foodborne viruses

Foodborne illnesses caused by viruses, bacteria, and parasites are a major concern for the food industry today. According to the latest estimations of foodborne illnesses in the United States, 31 pathogens are responsible for 9.4 million foodborne illnesses, 55,961 hospitalizations, and 1,351 deaths (Scallan et al., 2011). Of these, viruses are estimated to be the responsible for 59% of the total foodborne illnesses, 27% of the hospitalizations, and 12% of the deaths (Scallan et al., 2011).

The major difference between viruses, bacteria, and fungi is that viruses need a host cell to replicate. Foodborne viruses, especially enteric viruses, are typically spread by the fecal-oral route as a result of contaminated food, water, contact surfaces and people from poor sanitation practices by infected food handlers (Todd et al., 2008). Foodborne viruses have the potential to be shed by infected people before, during, or after symptoms occur, as well as by those who never show symptoms (Atmar et al., 2008; Parashar et al., 1998). Foods commonly contaminated with foodborne viruses include shellfish, fresh produce, fruits and juices, and other foods that do not undergo further thermal processing (Baert et al., 2009; Sair et al., 2002).

Some viruses have the ability to be environmentally stable and resistant to temperature, pH, and enzymes, such as enteric viruses (Jaykus et al., 2000; Scipioni et al., 2008). The leading cause of foodborne non-bacterial gastroenteritis is due to the human norovirus (Mead et al., 1999). In 1968, a school outbreak in Norwalk, Ohio resulted in infected school children spreading the infection to family members, which eventually led to an outbreak. The virus that was originally called the winter vomiting disease became known as the "Norwalk-like virus". In 1972, the virus was identified as a small round shaped virus (Kapikan et al., 1996). Due to advancements in technology, the morphology and phylogeny of the Norwalk-like virus are better understood and have resulted in name change to the human norovirus (Lopman et al., 2008). The *Norovirus* genus is one of two genera in the *Caliciviridae* family, which includes *Norovirus*, *Vesivirus*, *Lagovirus*, and *Sapovirus*, that cause gastroenteritis outbreaks (D'Souza et al., 2007). Human noroviruses are single stranded, non-enveloped, positive sense RNA viruses, and icosahedral in shape being about 27-38 nm in size (Green et al., 2001, Hyde et al., 2009; Taube et al., 2010). The RNA genome of the norovirus is about 7.5kb in size, which contains a poly A tail on the 3' end of the genome and a sequence that is 7,642 nucleotides long on the other end (Jiang and Estes, 1990, D'Souza et al., 2007; Scipioni et al., 2008).

The infection process of human noroviruses is not completely understood, but it is thought that the infection starts with the virus binding to histo blood group antigens (HBGAs), which are complex glycans on red blood cells in the gut, which initiates the replication process (Donaldson et al., 2008; Perry and Wobus, 2010). The infection consists of self-limiting symptoms, such as nausea, vomiting, diarrhea, and fever, which typically resolve within 3 days (Cliver et al., 2002; Grohmann et al., 1981; Grove et al.,

2006; Patel et al., 2008). Children, the elderly, and the immunocompromised are the most vulnerable people to a norovirus infection. Norovirus outbreaks occur mainly in closed settings, such as hospitals, cruise ships, military bases, and day cares, due to the ease of transmission through contamination (Widdowson et al., 2005). The ability of the virus to spread easily could be due to low infectious doses, which requires only 10-100 particles, length of shedding time, stability in a wide range of environments, and a lack of immunity developed by people (Cheesbrough et al., 2000, Jaykus et al., 2000, Teunis et al., 2008; Duizer et al., 2004). Human noroviruses have been detected for up to 7 days after inoculation on Formica, ceramic, and stainless steel, and found to have a 2.3 log or less reduction after 90 days in frozen storage on strawberries, raspberries, and blueberries (Butot et al., 2008; D'Souza et al. 2006).

In 2001, an outbreak of norovirus affecting 30 people in Sweden involved bakery products with contaminated raspberries (Le Guyader et al., 2004). In July 2005, a norovirus outbreak happened at a summer camp in California involving 15 children due to a food handler not practicing proper hygiene (Barrabeig et al., 2010). In November 2007, a Swedish manufacturing company had 413 workers with gastroenteritis due to 2 food handlers contaminating tomatoes with norovirus (Zomer et al., 2010). On a cruise ship in 2008, there was a norovirus outbreak involving 196 people as a result of improper personal hygiene, which was spread through person to person contact (Vivancos et al., 2010). In Spain, 59 students were involved in a norovirus outbreak as a result of contamination by food handlers (Godoy et al., 2005). As a result of a patron with a norovirus infection vomiting in a restaurant, there was an outbreak in which 52 people became sick (Marks et al., 2000). There have many outbreaks of norovirus worldwide

from 1995-2004 due to spread through water including pools, drinking water, fountains, tap water, and municipal water at hotels, recreation centers, restaurants, and in communities (Maunula, 2007).

1.8. Enteric viral surrogates

Human noroviruses are currently not cultivable in a laboratory (Duizer et al., 2004), which is why cultivable surrogates, such as feline calicivirus (FCV-F9), murine norovirus (MNV-1), and coliphage MS2 are used in infectivity assays.

Feline calicivirus (FCV) has been commonly used as a surrogate for human noroviruses due to their genetic similarity as they both belong to the *Caliciviridae* family (Bidawid et al., 2000; D'Souza et al., 2006). FCV is a positive sense single stranded RNA virus with a genome about 7.7 kb (Radford et al., 2006). It is a member of the *Vesivirus* genus and transmitted through the nasal, oral or conjunctival passage ways of cats (Radford et al., 2006). Cells infected with FCV present a characteristic cytopathic effect associated with cell rounding and membrane bulging due to an inhibition of cellular protein synthesis (Knowles et al., 1988; Willcocks et al., 2004). FCV causes moderate to severe acute oral and upper respiratory illness in cats, which can be characterized with oral ulcerations, and ocular and nasal drainage (Duizer et al., 2004; Hurley et al., 2004; Radford et al., 2006). It can also lead to lameness or limping disease and some more virulent strains have lead to death (Duizer et al., 2004; Pederson et al., 2000; Radford et al., 2006). FCV can be shed for up to 30 days, infective for up to a month in the environment, and is typically prevalent in areas with large numbers of cats, such as

animal shelters (Doultree et al., 1999; Radford et al., 2006). There are broad-spectrum antibiotics and live-attenuated and inactivated vaccines used to control the spread of FCV. The difference between FCV and the human norovirus is that FCV is a respiratory virus, which is not adjusted to the environmental conditions of the intestinal tract, such as low pH and high bile concentrations, versus the human norovirus being less susceptible to the intestinal tract conditions because it is an enteric virus (Duizer et al., 2004; Perry et al., 2009; Radford et al., 2007; Cannon et al., 2006).

A study found FCV to have a 3 log reduction after 24 and 8 hr at 37 and 56˚C, respectively (Duizer et al., 2004). Gulati et al. (2001) found a 2 log reduction of FCV after washing inoculated strawberries and lettuce for 10 min with water. Another study found bleach at 50 and 100 ppm to cause a 2.2 and 2.6 log reduction, respectively, on lettuce after 2 min (Allwood et al. 2004). Allwood et al. (2003) found a FCV titer of 10^9 log PFU/ml in tap water to have a 1 log reduction after 7.7, 5.7, and 3.0 days at 4, 25, and 37˚C, respectively. Su et al. (2010c) found high intensity ultrasound at 20 kHz at < 21˚C to cause a 2.67 log reduction and complete inactivation after 30 min to FCV in PBS with a 10^6 and 10^4 titer, respectively. A 10^5 titer of FCV was treated with PBS at pH 7.0, PBS at pH 2.6, orange juice at pH 3.8, orange juice at pH 3.4, cranberry juice at pH 2.6, and cranberry juice at pH 7.0 to find a 0.12, 1.67, 1.40, 4.29, ,5.02, and 5.02 log reduction, respectively (Su et al. 2010a). D'Souza and Su (2010) found 2% trisodium phosphate to be enough to completely inactivate both a low titer (10^5) and a high titer (10^7). The same study found 10% bleach, and 1 and 2% glutaraldehyde to cause complete inactivation on both high and low titers after 30 seconds (D'Souza and Su, 2010). A different study found 53 kDa chitosan to cause a 1.09, 2.09, and 2.83 log reductions on 10^7 titer and a

2.12, 2.56, and 4.21 log reduction on a 10^5 titer after 3 hrs at 0.175, 0.35, and 0.7% concentrations (Su et al., 2009).

More recently murine norovirus (MNV-1) has become cultivable in a lab and has become another surrogate used for single stranded RNA viruses (Bae and Schwab, 2008; Cannon et al., 2006). MNV-1 is another member of the *Caliciviridae* family and even more genetically similar to the human norovirus because it is in the *Norovirus* genus. Other similarities between MNV-1 and the human norovirus are the size (28-35 nm in diameter), icosahedral shape, transmission route, and the symptoms after infection, which includes diarrhea, fever, nausea, and abdominal pain (Green et al., 2001; Karst et al., 2003). Since MNV-1 is the only norovirus that replicates in cell culture, it provides the first chance to understand the relationship between mechanisms of norovirus replication (Wobus et al., 2006). Although MNV-1 was originally isolated from brain tissue in mice, this is not characteristic of the normal biology of the virus (Karst et al., 2003; Wobus et al., 2006). Other scientist have been unable to isolate the pathogen from the brain of wild type and immunocompromised lab mice that have been naturally infected or inoculated through the peroral and intranasal routes with MNV-1 (Karst et al., 2003; Wobus et al., 2006).

Over 2 hr MNV-1 had a 0.5-0.6 log reduction in a pH of 2- 4, even less of a reduction in titer between pH 5-9, and a ~1.8 log reduction at pH 10 (Cannon et al., 2006). This same study found that MNV-1 titer is reduced by 1 log at 56˚C, 63˚C (consistent with low-temperature, long-time pasteurization) and 72˚C (consistent with high-temperature, short-time pasteurization) in 6.7 min, 25 seconds, and 7 seconds, respectively (Cannon et al., 2006). The effect of different molecular weights chitosan on

MNV-1 showed that 53 kDa and 5 kDa chitosan reduced ~5 log or ~7 log PFU/ml viral titers MNV-1 by less than 0.5 log PFU/ml (Su et al., 2009). Another study found that washing inoculated onion bulbs and spinach leaves with water for 0.42 and 2 min, respectively, caused a 0.4 and 1.0 log reduction, respectively (Baert et al., 2008). A 10^5 titer of MNV-1 was treated with PBS at pH 7.0, PBS at pH 2.6, orange juice at pH 3.8, orange juice at pH 3.4, cranberry juice at pH 2.6, and cranberry juice at pH 7.0 to find a 0.07, 0.01, 0.03, 0.09, 2.06, and 1.64 log reductions, respectively (Su et al., 2010a). Su et al. (2010c) found high intensity ultrasound at 20 kHz at < 21˚C to caused a 0.07 and >3.79 log reduction after 30 min to FCV in PBS with a 10^6 and 10^4 titer, respectively. A study by D'Souza and Su (2010), found 5% TSP to be enough to completely inactivate a high titer of MNV-1 after 30 seconds, while 1 and 2% glutaraldehyde, 10% bleach, and 70% ethanol caused a 2.44,6, 2.52, and 0.0 log reduction, respectively (D'Souza and Su, 2010). The same study found 2% TSP, 1 and 2% glutaraldehyde, and 10% bleach to be enough to completely a low titer of MNV-1 after 30 seconds, while 70% ethanol still had no effect on the titer (D'Souza and Su, 2010).

Bacteriophage MS2, which is commonly found in sewage and adopted to the intestinal tract, is another enteric virus that is used as a surrogate for single stranded RNA viruses as it is resistant to environmental conditions and used for environmental studies (Dawson et al., 2005; Guan et al., 2006; Shin and Sobsey, 2003). As a member of the *Leviviridae* family, MS2 has some similarities to human noroviruses, which include being a positive sense, single stranded RNA viruses about 22-29 nm in size and icosahedral in shape (Calender, 1988; Dawson et al., 2003; Toropova et al., 2008). As a

bacteriophage, MS2 natural host is Gram negative bacteria, such as *E. coli* ATTC 15597B (Friedman et al., 2009).

Over 4 hrs MS2 demonstrates stability in a pH of 6.7, but has a 1.11 log reduction at pH 3.9, which is the isoelectric point (Langlet et al. 2007), and a 3 log reduction in a pH of 2.5 (Langlet et al. 2007). Other studies have found proanthocyanins and polyphenols of cranberry juice and pomegranate juice to be the major reason for a titer decrease of MS2 rather than the pH (Su et al., 2010a; Su et al., 2010b). A 10^5 titer of MS2 was treated with PBS at pH 7.0, PBS at pH 2.6, orange juice at pH 3.8, orange juice at pH 3.4, cranberry juice at pH 2.6, and cranberry juice at pH 7.0 to find a 0.00, 0.34, 0.13, 0.71, 1.14, and 0.39 log reductions, respectively (Su et al., 2010a). The effect of different molecular weights chitosan on MS2 showed that 53 kDa chitosan reduced ~5 log PFU/ml viral titers of bacteriophage MS2 by 1.70 log PFU/ml, which was more efficient than 5 kDa chitosan that had a 0.98 log PFU/ml reduction (Su et al., 2009).

D'Souza et al. (2010) found that 1% trisodium phosphate (TSP), which is a common household cleaner, with 30 s contact times decreased a high titer of MS2 by 4.5 logs PFU, while the 2% and 5% TSP caused complete inactivation of the high titer. The same study found 10% bleach, 70% ethanol, and 1 and 2% glutaraldehyde to cause complete inactivation, 0.06, 2.18, and 3.22 log PFU/ml reduction on a high titer after 30 s (D'Souza et al., 2010). Dawson et al. (2005) found 100 ppm chlorine for 5 min to cause a 0.7 log reduction of MS2 on lettuce. Another study found that 20 ppm chlorine for 10 min caused a greater than 1.8 log reduction on lettuce (Casteel et al., 2008). One study found that 50 and 100 ppm caused a 1.9 and 2.7 log reduction, respectively, on lettuce after two min (Allwood et al., 2004). Dawson et al. (2005) did a study on the ability of

MS2 to survive on fresh iceberg lettuce, baton carrot, cabbage, spring onion, curly leaf parsley, capsicum pepper, tomato, cucumber, raspberries, and strawberries, which found that MS2 had less than a 1 log reduction after 50 days at 4 and 8°C. Allwood et al. (2003) found a titer of 10^9 log PFU/ml MS2 in tap water to take 25.7, 18.7, and 2.7 days at 4, 25, and 37˚C, respectively, to have a 1 log reduction. Another study found that high intensity ultrasound completely inactivated a ~10^4 titer of MS2 in PBS after 10 min and caused a 4.62 log reduction on the high titer of 10^6 in PBS after 30 min (Su et al., 2010c).

Phi X174 is a member of the *Microviridae* family and a bacteriophage used as a surrogate for single stranded DNA enteric viruses (Brentlinger et al., 2002). Although phi X174 is different from the other surrogates because it is a positive sense, circular DNA virus, it is about 30 nm in size, icosahedral in shape, and has about a 4.4-6.3 kb genome (Bennett et al., 2008; Ilag et al., 1994; Suzuki et al., 1999; Wichman and Brown, 2010). Phi X174 starts the infections process by attaching to a lipopolysaccharide on the host cell surface (Bennett et al., 2008; Bernhardt et al., 2002). This virus can typically be found in soil, seawater, sewage, and in the intestine of animals (Brentlinger et al. 2002). Phi X174 has an isoelectric point of 6.6 (Helmi et al., 2008) and infects Gram negative bacteria including *E. coli*, *S.* Typhimurium and *Shigella sonnei*.

A10^5 titer of phi X174 was treated with PBS at pH 7.0, PBS at pH 2.6, orange juice at pH 3.8, orange juice at pH 3.4, cranberry juice at pH 2.6, and cranberry juice at pH 7.0 to find a 0.00, 0.00, 0.37, 1.01, 1.79, and 0.93 log reductions, respectively (Su et al., 2010). Another study found that at 55% relative humidity it takes 1.87 and 0.85 ppm ozone to cause 90% inactivation after 13.8 and 18.4 s, respectively (Tseng and Li, 2006).

Solomon et al. (2009) found phi X174 to be completely resistant to quaternary ammonium compounds and very susceptible to oxidative disinfectants, such as virkon.

Therefore, these four viruses were selected to determine the effectiveness of chitosan as an antiviral agent against single stranded RNA and DNA viruses. The objective of this study was to determine the effect of different molecular weights and concentrations of chitosans on these enteric viral surrogates FCV-F9, MNV-1, phi X174 and MS2.

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CHAPTER III

Effectiveness of chitosan on the inactivation of enteric viral surrogates

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Abstract

Chitosan is known to have bactericidal and antifungal activity. Although human noroviruses are the leading cause of nonbacterial gastroenteritis, information on chitosan efficacy against food-borne viruses is very limited. The objective of this work was to determine the effectiveness of chitosans of different molecular weight against the cultivable human norovirus surrogates, feline calicivirus, FCV-F9, murine norovirus, MNV-1, and bacteriophages, MS2 and phi X174. Five purified chitosans (53, 222, 307, 421, ~1,150kDa) were dissolved in water, 1% acetic acid, or aqueous HCl pH= 4.3, sterilized by membrane filtration, and mixed with equal volume of virus suspension to obtain a final concentration of 0.7% chitosan and ~5 log PFU/ml. Virus-chitosan suspensions were incubated for 3 hr at 37°C. Untreated viruses in PBS, in PBS with acetic acid, and in PBS with HCl pH=4.3 were tested as controls. Water-soluble chitosan (53 kDa) reduced phi X174, MS2, FCV-F9 and MNV-1 titers by 0.59, 2.44, 3.36, and 0.34 log PFU/ml respectively. Chitosans in acetic acid decreased phi X174 by 1.19-1.29, MS2 by 1.88-5.37, FCV-F9 by 2.27-2.94, and MNV-1 by 0.09-0.28 log, respectively. Increasing the molecular weight of chitosan showed a greater effect on MS2, but for all other surrogates it did not appear to play a role on the antiviral effect of chitosan. Overall, chitosan treatments showed a greatest log reduction of MS2, followed by FCV-F9, phi X174, and with no significant effect on MNV-1.

1. Introduction

Chitosan has shown vast potential as an antimicrobial additive. It is a cationic polysaccharide composed of glucosamine and acetyl-glucosamine units bound by b-(1→4) glycosidic bonds, and classified by its molecular weight (MW) and degree acetylation (DA). Generally, MW of chitosan ranges between 50 – 1,000 kDa with DA between 0 - 30%. Chitosan is not currently recognized as GRAS, but it has acquired EPA approval to be used as a biopesticide (Docket No. EPA-HQ-OPP-2007-0566-0019). Preparations with chitosan are presently applied in the fields for waste water management as a chelator, in medicine as wound dressings, and in agriculture as fungicidal sprays (Koide, 1998; Reddy et al., 2000; Shahidi et al., 1999). Chitosan has shown promise for use in the food industry as an antimicrobial, antioxidant, clarifying agent, and as a packaging material (Devlieghere et al., 2004; Shahidi et al., 1999; Xie et al., 2001). It has proven microbiocidal activity against bacteria and fungi (Helander et al., 2001; Reddy et al., 2000) and recent data indicate it may have antiviral effects as well (Su et al., 2009). The molecular weight, degree of acetylation, and the concentration are the factors that determine the antimicrobial efficiency of chitosan (Chirkov, 2002).

Although the mechanisms of chitosan antimicrobial action are not completely understood, there are several hypothesized mechanisms based on the positive charge of the NH_3^+ group below a pH of 6.3 (Liu et al., 2004). Chitosan is thought to control bacterial growth by one or more mechanisms including leakage of intracellular material by interacting with the negative charges of the outer cell surface, blocking nutrient

transport to and from the cell by stacking onto the cell surface, and interfering with DNA transcription (No et al., 2002; Rabea et al., 2003; Tharanathan and Kittur, 2003; Zheng and Zhu, 2003). Applied to fungi and yeast, chitosan is thought to interact with DNA causing the inhibition of RNA and protein synthesis, increase membrane permeability by interacting with charged phospholipids or proteins in the cellular membrane, and reduce growth due to chelation of Ca^{2+} and other essential minerals or accumulation in the cell affecting the cell wall and hyphae growth (Durango et al., 2006; El Ghaouth et al., 1992; Hadwiger and Loschke, 1981; Roller and Covill, 1999). The proposed antiviral mechanisms include blocking viral replication, and neutralizing mother and daughter phage particles by affecting a stage of reproduction or neutralizing the virulence (Chirkov, 2002; Kochkina and Chirkov, 2000). In addition, it has been shown that chitosan acts indirectly in plants by inducing defense mechanisms towards various plant pathogens including bacteria, fungi, and viruses (Badawy and Rabea, 2009; Fajardo et al., 1998; Liu et al., 2007).

Human noroviruses are enteric viruses that are considered as the leading cause of foodborne non-bacterial gastroenteritis (Mead et al., 1999). They belong to the *Caliciviridae* family and are single stranded, non-enveloped, positive sense RNA viruses (Donaldson et al., 2008; Hyde et al., 2009) Human noroviruses are icosahedral in shape and about 27-38 nm in size (Donaldson et al., 2008; Taube et al., 2010). They are thought to infect host by binding to histo blood group antigens (HBGAs) or complex glycans on red blood cells in the gut, to iniate cell entry replication (Donaldson et al., 2008; Perry and Wobus, 2010). Only 10 -100 viral particles are thought to be capable of causing

infection and they are highly resistant to environmental conditions (Cheesbrough et al., 2000).

Since human noroviruses are currently not cultivable in a lab (Duizer et al., 2004), cultivable surrogates, such as feline calicivirus, FCV-F9; murine norovirus, MNV-1; and coliphage MS2, are used in infectivity assays. Feline calicivirus F9 (FCV-F9) has been commonly used as a surrogate for human noroviruses because it also belongs to the same *Caliciviridae* family as human noroviruses (Bidawid et al., 2000; D'Souza et al., 2006). Feline calicivirus differs from human noroviruses because it is a respiratory virus that is more susceptible to environmental conditions, such as low pH, than human noroviruses (Cannon et al., 2006; Duizer et al., 2004; Perry et al., 2009; Radford et al., 2007;). On the other hand, the recently cultivable murine norovirus (MNV-1) is more resistant to environmental conditions than FCV-F9, is shed in feces, and is therefore considered to a better surrogate for human noroviruses by some researchers (Bae and Schwab, 2008; Cannon et al., 2006; Su et al., 2009; Wobus et al., 2006). MS2 is a bacteriophage that is used as a surrogate for RNA viruses in environmental studies because it is resistant to environmental conditions (Dawson et al., 2005; Guan et al., 2006; Shin and Sobsey, 2003). MS2 is a member of the *Leviviridae* family, similar to human noroviruses in being positive sense, single stranded RNA viruses that are about 22-29 nm in size with an icosahedral shape (Dawson et al., 2003; Langlet et al., 2007; Toropova et al., 2008). Phi X174 is a bacteriophage used as a surrogate for single stranded DNA enteric viruses. Phi X174 is a member f the *Microviridae* family and is a positive sense, circular, single-stranded DNA bacteriophage, about 30 nm in size (Brentlinger et al., 2002; Ilag et al., 1994; Suzuki et al., 1999). It has an icosahedral shape and infects it host by attaching to a

lipopolysaccharide on the host cell surface (Bennett et al., 2008; Bernhardt et al., 2002). Therefore, these four viruses were chosen to determine the effectiveness of chitosan as an antiviral agent.

The objective of this study was to determine the effect of different molecular weight chitosans on the infectivity of enteric virus surrogates FCV-F9, MNV-1, phi X174 and MS2.

2. Materials and Methods

2.1. Viruses, hosts, and cell lines

Coliphage phi X174 and its host *Escherichia coli* CN-13 (both received as a gift from Dr. Suresh Pillai of Texas A&M University, College Station, TX); Coliphage MS2 and host, *E. coli* B-15597 (both from ATCC, Manassas, VA); Feline Calicivirus F9 (FCV-F9) and cell line Crandell Reese Feline Kidney (CRFK) cells (both from ATCC, Manassas, VA); Murine Norovirus-1 (MNV-1; graciously provided by Dr. Skip Virgin, Washington University, St. Louis, MO) and host RAW 264.7 cells (from the collection of University of Tennessee at Knoxville) were used in this study.

2.2. Virus Propagation

For phi X174 and MS2, hosts *E. coli* CN-13 and *E. coli* B-15597 respectively, were transferred twice in a 3% trypticase soy broth containing 0.1% glucose, 20µg/ml

$CaCl_2$, and 10 µg/ml thiamine with an incubation period of 6 hr at 37°C. After the second incubation, the viruses were added to their hosts for ~18 hr. The viruses were harvested by centrifugation at 3,000 x g for 10 min and filtered through a 0.2 µm membrane filter. Finally, 1 ml aliquots placed into vials and the viruses were stored frozen at -20°C until use in the experiment. For MNV-1 and FCV-F9, host cells Raw 264.7 and CRFK, respectively, were incubated at 37°C with 5% CO_2. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin-streptomycin and heat inactivated 10% fetal bovine serum (FBS). CRFK and RAW 264.7 cells were infected with FCV-F9 and MNV-1, respectively, and incubated at 37°C with 5% CO_2 until 90% lysis or greater. The incubation time for FCV-F9 was ~24 hrs and for MNV-1 was ~4-6 days. After lyses, the viruses were harvested by freeze-thawing, once for FCV-F9 and three times for MNV-1. The viruses were centrifuged at 5,000 x g for 10 min. Finally, supernatants were filtered through a 0.2 µm membrane filters, 1 ml aliquots placed into vials, and frozen at -80°C until use in the experiment.

2.3. Chitosan application for inactivation

Water-soluble chitosan (MW 53 kDa, DA 9.1%; EZ Life Science Co. Ltd., Seoul, South Korea) dissolved in sterile deionized distilled water to obtain a concentration of 1.4%, was mixed with equal volume of virus suspension in PBS to obtain a final concentration of ~5 $\log_{10}$ PFU/ml virus titer and a 0.7% concentration of chitosan, and incubated for 3hr at 37°C. Each of four other chitosans (222kDa, 32.5%DA, as determined in our lab, Primex, Iceland; 307kDa, 20.2%DA, as determined in our lab,

Primex, Iceland; 421kDa, 30.3%DA, as determined in our lab, Primex, Iceland; high molecular weight, max 25%DA with an estimated MW of ~1,150kDa, Aldrich, St. Louis, MO) was dissolved in 1% acetic acid and sterile deionized distilled water to form a chitosan concentration of 1.4%. Chitosan solutions were mixed with equal volume of virus suspension in PBS to reach a mixture of ~5 $\log_{10}$ PFU/ml virus titer and 0.7% chitosan in 0.5% acetic acid, and incubated for 3hrat 37°C. Five controls were applied: (a) "3 hr" - a 3 hr incubation virus control (~5 $\log_{10}$ PFU/ml in PBS), (b) "AcAc4.5" - a virus control (~5 $\log_{10}$ PFU/ml in PBS) with acetic acid (~0.15%) to have pH similar to the pH of 222-1,150 kDa chitosan and virus mixture, (c) "AcAc5.6" - a virus control (~5 $\log_{10}$ PFU/ml in PBS) with acetic acid (~0.0625%) to have pH similar to the pH of 53 kDa chitosan and virus mixture, (d) "HCl4.5" - a virus control (~5 $\log_{10}$ PFU/ml in PBS) with hydrochloric acid (~0.010125%) to have pH similar to the pH of 222-1,150 kDa chitosan and virus mixture and (e) "HCl5.6" - a virus control (~5 $\log_{10}$ PFU/ml in PBS) with hydrochloric acid (~0.0095%) to have pH similar to the pH of 53 kDa chitosan and virus mixture. To eliminate effect of acid used as a solvent, 222 - ~1,150 kDa chitosans were tested in acetic acid and in aqueous HCl at pH values similar to the pH of the chitosan in acetic acid samples (pH 4.15 – 4.3). All treatments were done in duplicate and replicated at least twice.

2.4. Plaque Assay Infections

The method of Bae and Schwab (2008) and Su et al. (2009) was followed for the MS2 and phi X174 plaque assays with the exception that 0.7 ml of serially diluted treated

or untreated phage MS2 was mixed with 0.3 ml of 5-6 hr *E. coli* B-15597 in 8 ml of 0.6 % tryptic soy top agar, and poured on tryptic soy agar (TSA) plates containing 0.5% NaCl. For phi X174, 0.7 ml of serially diluted treated or untreated phage phi X174 was mixed with 0.25 ml of 5-6 hr *E. coli* CN-13 host with 8 ml of 0.6 % trptic soy top agar, and poured on TSA plates. For both viruses, plates were incubated at 37^{o}C overnight and plaques were counted.

Plaque assays for MNV-1 were done similarly to the procedures described by Wobus et al. (2004) and Su et al. (2009). After flasks containing RAW 264.7 cells were confluent, 2 ml of cells were added to each well in a 6-well plates and incubated until ~90% confluent. Treated and untreated MNV-1 was serially diluted tenfold in DMEM-F12 containing 10% FBS. After the media of each 6-well plate was aspirated, 0.5 ml of each virus dilution (treated and untreated controls) was inoculated and cells were incubated at 37°C under 5% CO_2 for 2.5 hr for infection. Again, the media of the 6-well plates was aspirated and the cells were overlaid with 2 ml of DMEM F-12 containing 0.75% agarose, 10% FBS and 1% penicillin-streptomycin. The 6-well plates were allowed to solidify and then incubated for 72 hr at 37^{o}C under CO_2. Finally, the plates were overlaid with a staining media that contained the same ingredients as the first overlay media plus 0.02% neutral red, allowed to solidify, incubated for 3-5 hrs at 37°C under 5% CO_2 and plaques were counted.

Plaque assays for FCV-F9 were done according to the procedure of D'Souza et al. (2006) and Su et al. (2009). CRFK cells were grown in flask at 37°C under 5% CO_2 until confluent. CRFK cells were added to 6-well plates (2 ml) and incubated until ~90% confluent. Treated and untreated FCV-F9 were serially diluted tenfold in DMEM F-12

containing 2% FBS. After the wells in the plate were aspirated, they were inoculated with 0.5 ml of the virus dilutions (treated and untreated controls) and incubated for 2.5 ml at 37°C under 5% CO_2. The media of the 6-well plates were aspirated and then overlaid with DMEM F-12 containing 0.75% agarose, 2% FBS and 1% penicillin-streptomycin. The plates were incubated for 48 hrs and overlaid with overlay media that contained 0.01% neutral red along with the other ingredients of the previous overlay. The plates are allowed to solidify, incubated for no longer than 24 hrs under 5% CO2, and plaques were counted.

2.5. Statistical analysis

ANOVA and Tukey's test were determined on a completely randomized design with sampling using SAS software (version 9.2, SAS Institute, Cary, NC, USA) on data from at least two replications with duplicates.

3. Results and Discussion

3.1 Acetic acid and HCl effect on viruses

The pH of the chitosan solutions did cause a significant reduction on most of the tested surrogates at 37˚C for 3 hr. Phi X174 exhibited a 0.70-0.69 and 0.46-0.47 log PFU/ml reduction at pH 4.5 and 5.6, respectively (Table 1). Our results showed a 0.89-1.02 and 0.58-0.64 log PFU/ml reduction at pH 4.5 and 5.6, respectively, for MS2 (Table

1). In our study FCV-F9 had a 0.91-0.95 and 0.38-0.47 log PFU/ml reduction at pH 4.5 and 5.6, respectively (Table 1). For MNV-1, we found that 0.19-0.23 and 0.30-0.14 log PFU/ml reduction at pH 4.5 and 5.6, respectively (Table 1).

Controls with acetic acid and hydrochloric acid alone were run to confirm that reduction in the virus titer was due to the chitosan rather than the pH and/or type of acid in the chitosan solutions. Except for 222 kDa against MS2 and FCV-F9, the type of acid did not have an effect on the antiviral activity. Technical difficulties encountered during preparation of high viscosity chitosans at high concentration could potentially account for the differences in viral recovery between replicates and acetic acid and hydrochloric acid. Acetic acid is the typical acid used with chitosan and is known to have antibacterial activity. Thus, hydrochloric acid was used at the same pH as acetic acid samples. Acetic acid has been found to be effective in inactivating microorganisms that include *Listeria monocytogenes, Enterobacter sakazakii,* and *Salmonella enterica* serovar Typhimurium (Alvarez-Ordonez et al., 2009; Back et al., 2009; Vasseur et al., 1999). However, hydrochloric acid has been shown to have limited antibacterial activity compared to acetic acid on *Listeria monocytogenes*, *Enterobacter sakazakii,* and *Listeria innocua* (Back et al., 2009; Conner et al., 1989; Ita and Hutkins, 1991; Miller et al., 2009). Hydrochloric acid has less antibacterial activity because it has a higher dissociation constant than acetic acid. Undissociated acetic acid is able to enter the bacteria cell, dissociate and decrease the pH inside the cell, and cause the cell to use up its energy trying to remove the hydrogen ions.On the other hand, HCl is dissociated at all practical pH (pH >1), and thus unable to easily pass the cell membrane.Our results had similar reduction trends to previous studies showing the effect of pH on MS2. Langet et al.

(2007) found a 1.11 log PFU reduction and no reduction on MS2 at pH 3.9 and 6.7, respectively. Our results for FCV-F9 were slightly lower than the results found in Cannon et al.(2006), who showed ~2.0, ~1.0, ~1.5 log reduction at pH 4, 5 and 6, respectively, after 30 min with citrate or phosphate buffer. A study by Cannon et al. (2006) found ~0.50, ~0.50, and ~0.10 log reduction at pH 4, 5, and 6, respectively, of MNV-1. The study by Cannon et al. (2006) tested 30 min and 2 hr, but chose to only present the 30 min results because a statistical difference was not seen. Differences in the inactivation trends at the pH values between 4-6 could also be due to the different acids used in experiments. Citric acid and acetic acid have been shown to have similar antibacterial effects against *E. coli, S.* Typhimurium, *Y. enterocolitica,* and *L. monocytogenes,* which could be due to citric acid acting as a chelator (Akbas and Olmez, 2007; Dickson, 1992; Fernandez Escartin et al., 1989; Karapinar and Gonul, 1992). It could be possible in this case, that citric acid is more effective against these viruses than acetic acid.

3.2 Effect of Chitosan of phi X174

Chitosan with MW greater than 53 kDa reduced the recovery of phi X174 compared to the 3 hr control, but not to the pH controls (Table 1). Although the reduction was statistically significant ($P<0.05$), the greatest reduction was 1.29 log PFU/ml, which was achieved with ~1,150 kDa in acetic acid, out of which 0.70 log PFU/ml can be attributed to the effect of pH. The type of acid did not appear to have any effect on the virus recovery neither in controls nor in samples, but the pH of the solutions did appear to be a factor in the reduction of the virus. Chitosan with the smallest molecular weight

tested (53kDa) had no effect on phi X174 recovery (0.59 log PFU/ml), while all other chitosans resulted in titer reduction of 1.06-1.29 log PFU/ml.

3.3 Effect of Chitosan on MS2

Chitosan, in general, had the largest effect on MS2, followed by FCV-F9 and phi X174, and had minimal to no effect on MNV-1 (Table 1). The recovery of MS2 compared to the controls was significantly reduced by all five tested chitosans (Table 1). Chitosans with MWs from 53 to 307 kDa had similar effects on MS2, and further increase in MW resulted in further reduction of MS2 recovery. Both acetic (pH 5.6) and hydrochloric acid (pH4.5) controls caused less than a log PFU/ml reduction (0.61 and 0.96, respectively). Chitosan of 222 kDa appeared to be more effective in HCl than in acetic acid, resulting in 3.27 and 2.28 log PFU/ml reduction, respectively. Chitosan with the highest tested molecular weight (~1,150 kDa) caused the greatest reduction in MS2, resulting in complete inactivation. For MS2, increase in molecular weight of chitosan was directly proportional to the reduction in MS2 titers.

3.4 Effect of Chitosan on FCV-F9

FCV-F9 was similarly affected by both acids at both pH values, which was about 0.43 and 0.93 log PFU/ml reduction at pH 5.6 and 4.5, respectively (Table 1). Chitosan of all five molecular weights did cause significant decreases in the recovery of FCV-F9 compared to the controls. Contrary to effects on MS2, there was no indication that

molecular weight of chitosan has any effect on its antiviral activity against FCV-F9. Interestingly however, 222 kDa chitosan in HCl was more efficient than the same chitosan in acetic acid against both MS2 and FCV-F9.

3.5 Effect of Chitosan on MNV-1

The recovery of MNV-1 was not affected by any of the five tested chitosans (Table 1). In addition, neither acetic nor hydrochloric acid at pH 4.5 or 5.6 had any effect on the virus infectivity. The reduction values ranged between 0.09 and 0.34 logs PFU/ml, for 307 kDa in acetic acid and 53 kDa chitosan, respectively. Longer contact time between chitosan and virus or higher concentrations of chitosan may be needed to inactivate MNV-1.

3.6 Discussion of chitosan as an antiviral agent

The proposed direct antiviral mechanisms of chitosan are similar to the antifungal and antibacterial mechanisms. The hypotheses are that chitosan causes structural damage to the virus causing inactivation (Kochkina et al., 2000), interacts with the negative charge of the viral capsids (Kochkina et al., 2000; Su et al., 2009), inhibits phage infection (Kochkina et al., 1995), or interferes with a step in the replication process (Chirkov, 2002; Kochkina et al., 2000). Chitosan has been shown to exhibit indirect antiviral activity by penetrating the plant cell to induce resistance (Kulikov et al., 2006).

However, there is not much literature on the effect of chitosan on foodborne viruses (Su et al., 2009).

Molecular weight is thought to play an important role in the antibacterial effect of chitosan. Studies have found the antibacterial activity to increase against Gram negative (*Escherichia coli*) bacteria as the MW decreases (Li et al., 2009; Zheng and Zhu, 2003). Also, Zheng and Zhu (2003) studied the effect of different molecular weights on Gram positive bacteria (*Staphylococcus aureus*) and found that as molecular weight increased from less than 5kDa to 305 kDa the antibacterial activity increased. Similarly, chitosan between 28 – 1671 kDa inhibited growth of both Gram negative (*E. coli, Pseudomonas fluorescens, Salmonella* Typhimurium, and *Vibrio parahaemolyticus*) and Gram positive (*Listeria monocytogenes, Bacillus cereus, S. aureus, Lactobacillus plantarum, Lactobacillus brevis,* and *Lactobacillus bulgaricus*) bacteria better than chitosan oligomers between 1-22 kDa (Gerasimenko et al., 2004; Zivanovic et al., 2004).

Effect of molecular weight of chitosan against viruses has mostly been studied indirectly, as chitosan-induced plant resistance to viral attack. Thus, Chirkov et al. (1998) reported that as the molecular weight of chitosan increased from 3-50 kDa, bean plants resistance to bean mild mosaic virus increased, but Kulikov et al. (2006) found that bean plants resistance to bean mild mosaic virus decreased as the MW of chitosan increased from 1.2-40.4 kDa. The only recently published study on the direct effects of chitosan of different molecular weights on human noroviruses surrogates showed that 53 kDa chitosan reduced ~5 log PFU/ml viral titers of bacteriophage MS2 and FCV-F9 (1.70 and 4.21 log PFU/ml, respectively) more efficiently than 5 kDa chitosan (0.98 and 1.41 log PFU/ml, respectively) (Su et al. 2009). Between Su et al. (2009) and this study, 0.7%

chitosan ranging from 5kDa to ~1,150 kDa is found to be ineffective at inactivating MNV-1 after 3hr contact.

Several chemical methods and additives have been studied to determine their effect on these enteric viral surrogates The titer of MS2 was completely inactivated (6.98 log PFU/ml) by 5% TSP (trisodium phosphate) and 10% bleach, while a 4.90, 6.03, 2.15, and 3.74 PFU/ml log reduction was seen for 1 and 2% TSP and 1 and 2% glutaraldehyde, respectively after 1 min contact time (D'Souza and Su, 2010). In our study, after 3 hr incubation, chitosan between 53-~1,150 kDa chitosan caused between a 1.85-5.37 (complete inactivation) log PFU/ml reduction. Similarly, a titer of ~10^5 log FCV-F9 was found to be completely inactivated by 1% TSP 1% glutaraldehyde, and 10% bleach using a 1 minute of contact time (D'Souza and Su, 2010) while we showed thtat chitosan between 53 and ~1,150 kDa decreased the titer of ~10^5 FCV-F9 between 2.70-4.31 log PFU/ml after 3 hr.

A titer of ~10^7 log PFU/ml MNV-1 was completely inactivated by 5% TSP, nearly completely inactivated by 2% glutaraldehyde (6 log reduction), and reduced by ~3 log PFU/ml by 1% glutaraldehyde and 10% bleach while 1 and 2% TSP had a ~1 log reduction or less. Liquid hydrogen peroxide at 2.1% was found to cause a ~3 and ~4 log PFU/ml reduction of MNV-1 and phi X174, respectively after 10 min (Li et al., 2011). The present study shown that chitosan between 53-~1,150 kDa reduced ~10^5 phi X174 by 0.59-1.29 log PFU/ ml and did not significantly decrease the titer of ~10^5 MNV-1 at any of the tested MWs. Chitosan used solely to clean contaminated products does not appear to be as sufficient antiviral agent compared to these other chemicals. However, chitosan does still show potential as a natural antiviral while being used for other potential

applications in the food industry where antibacterial and antifungal properties are needed as well as antioxidant, chelating, clarifying, or thickening properties, such as pre- or post-harvest sprays, water purification, additives, or packaging.

Bacteriophage MS2 showed the most vulnerability to chitosan. It showed similar susceptibility to chitosan from 53 kDa- 307 kDa, with an increase in antiviral activity as the molecular weight increased to ~1,150 kDa. There is a larger gap in MW range between 421 kDa and ~1,150 kDa compared to 53 kDa - 307kDa, which could be the reason for the observed increase in the antiviral activity with ~1,150kDa for MS2. For FCV-F9 and phi X174, chitosan showed statistically similar effects over the entire chitosan MW range used in the experiment and did not show a trend between the molecular weight and antiviral effect. MNV-1 showed no significant reduction in titers due to chitosan treatment. Although, a clear trend of increasing antiviral activity with increasing molecular weight cannot be seen for all the four viruses tested, lower molecular weight chitosan does not appear to be more effective than the higher molecular weight chitosan on any of the tested viruses. The method of viral inactivation by chitosan needs to be examined to help explain the inactivation of viruses seen in this study.

4. Conclusions

Chitosan, at a concentration of 0.7%, was the most effective against MS2, followed by FCV-F9 and phi X174, while ineffective against MNV-1. Reduction of MS2 infectivity by chitosan increased as molecular weight of chitosan increased, with high molecular chitosan (~1,150 kDa) being able to completely reduce the virus titer.

Susceptibility of FCV-F9 and phi X174 was not MW-dependent. In addition, the pH of the medium had more effect on the infectivity of the tested viruses than the type of the acid used to lower th pH, except in the case with 222 kDa chitosan in acetic and hydrochloric acid against MS2 and FCV-F9,. This data indicates that chitosan as an antiviral agent has potential application in the food industry, which could be in packaging material, coatings, or as pre- or post-harvest sprays on crops.

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Appendix

Table 1. Effect of 0.7% chitosan in water, acetic acid (AcAc) or hydrochloric acid (HCl) on the reduction of phi X174 phage, MS2 phage, FCV-F9, and MNV-1 using titers of ~5 log PFU/ml. Different letters when compared within each column denote significant differences ($P<0.05$).

Treatment		Reduction (log PFU/ml)			
		Phi X174	MS2	FCV-F9	MNV-1
3 hr control		$0.00^{C} \pm 0.00$	$0.00^{F} \pm 0.00$	$0.00^{D} \pm 0.00$	$0.00^{A} \pm 0.00$
5.6 pH	AcAc cont.	$0.47^{BC} \pm 0.08$	$0.64^{E} \pm 0.09$	$0.47^{D} \pm 0.04$	$0.03^{A} \pm 0.04$
	HCl cont.	$0.46^{BC} \pm 0.07$	$0.58^{EF} \pm 0.11$	$0.38^{D} \pm 0.04$	$0.14^{A} \pm 0.04$
	53 kDa	$0.59^{ABC} \pm 0.02$	$2.44^{D} \pm 0.14$	$3.84^{AB} \pm 0.68$	$0.34^{A} \pm 0.36$
4.5 pH	AcAc cont.	$0.70^{ABC} \pm 0.07$	$1.02^{E} \pm 0.10$	$0.95^{D} \pm 0.08$	$0.19^{A} \pm 0.08$
	HCl cont.	$0.69^{ABC} \pm 0.14$	$0.89^{E} \pm 0.06$	$0.91^{D} \pm 0.07$	$0.23^{A} \pm 0.04$
	AcAc 222 kDa	$1.19^{AB} \pm 0.31$	$2.28^{D} \pm 0.25$	$2.49^{C} \pm 0.57$	$0.28^{A} \pm 0.28$
	HCl 222 kDa	$1.08^{AB} \pm 0.42$	$3.27^{C} \pm 0.40$	$4.31^{A} \pm 0.41$	$0.13^{A} \pm 0.11$
	AcAc 307 kDa	$1.20^{AB} \pm 0.28$	$1.88^{D} \pm 0.19$	$2.27^{C} \pm 0.43$	$0.09^{A} \pm 0.08$
	HCl 307 kDa	$1.15^{AB} \pm 0.28$	$1.85^{D} \pm 0.42$	$3.12^{ABC} \pm 0.15$	$0.14^{A} \pm 0.12$
	AcAc 421 kDa	$1.28^{A} \pm 0.27$	$3.90^{B} \pm 0.24$	$2.94^{BC} \pm 0.24$	$0.15^{A} \pm 0.09$
	HCl 421 kDa	$1.29^{A} \pm 0.25$	$3.56^{BC} \pm 0.35$	$2.84^{BC} \pm 0.10$	$0.18^{A} \pm 0.07$
	AcAc ~1,150 kDa	$1.29^{A} \pm 0.27$	$5.37^{A} \pm 0.00$	$2.70^{BC} \pm 0.08$	$0.20^{A} \pm 0.16$
	HCl ~1,150 kDa	$1.06^{AB} \pm 0.15$	$5.37^{A} \pm 0.00$	$3.17^{ABC} \pm 1.02$	$0.27^{A} \pm 0.22$

CHAPTER IV

Effectiveness of different concentrations of chitosan on the inactivation of enteric viral surrogates

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Running Head: Effectiveness of different concentrations of chitosan on the inactivation of enteric viral surrogates

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Abstract

Enteric viruses are a major problem in the food industry, especially the human noroviruses, which are the leading cause of nonbacterial gastroenteritis. Chitosan has been shown to be effective against some enteric viral surrogates, but a more comprehensive study is needed. The objective of this work was to determine the effect of chitosan concentration on the cultivable enteric viral surrogates, feline calicivirus, FCV-F9, murine norovirus, MNV-1, and bacteriophages, MS2 and phiX174. Two chitosans (53 and 222 kDa) were dissolved in water and 1% acetic acid, sterilized by membrane filtration, mixed virus suspension to obtain a final concentration ~5 log PFU/ml virus titer and 0.7%, 1.0%, 1.25, and 1.5% chitosan, and incubated for 3 hr at 37°C. Untreated viruses in PBS were tested as controls. The 53 kDa chitosan with tested concentrations reduced phi X174, MS2, FCV-F9 and MNV-1 titers by 0.68-0.94, 2.61-2.80, 2.64-2.91, and 0.10-0.35 log PFU/ml, respectively, while reduction by 222 kDa chitosan was 0.51-0.75, 2.63-5.16, 2.22-2.41, and 0.82-0.95 log PFU/ml, respectively. There was no significant improvement in reduction of phi X174, FCV-F9, and MNV-1 titers with increasing concentration of both chitosans in the tested range (0.7-1.5%). However, 1% 222kDa chitosan in acetic acid (pH 4.5) caused complete reduction (5.16 log) of MS2, while the reduction was only 2.63 when applied at 0.7%. Overall, chitosan treatments showed the greatest log reduction of MS2, followed by FCV-F9, phi X174, and MNV-1.

1. Introduction

The antimicrobial properties of chitosan against bacteria and fungi have been explored extensively, but there is still much to learn about viral inactivation. Chitosan, derived from chitin through deacetylation, is a positively charged polysaccharide composed of glucosamine and acetyl-glucosamine. It is classified by its molecular weight (MW), which mostly ranges between 50 to 1000 kDa, and degree deacetylation (DDA), which ranges between70-100%. Concerns of chitosan being an allergen have kept it off the GRAS (generally recognized as safe) list. However, studies have shown that chitosan should not be considered as an allergen because of the harsh acid and base steps in the extraction and deacetylation process, which remove proteins, fats, and any other impurities that may be present (Muzzarelli, 2010). Even though it is not GRAS, it has acquired EPA approval for use in biopesticides (Docket No. EPA-HQ-OPP-2007-0566-0019). There are numerous potential applications for chitosan in agriculture as an antioxidant, antimicrobial, clarifying agent, and as a packaging material (Devlieghere et al., 2004; Shahidi et al., 1999; Xie et al., 2001).

Chitosan has shown to have antimicrobial activity against bacteria, fungi, and yeast (Helander et al., 2001; Reddy et al., 2000; Sudarshan et al., 1992) and recent studies indicate it may have antiviral activity as well (Chirkov, 2002; Su et al., 2009). The MW, DDA, and the concentration are the features of chitosan associated with the antimicrobial activity (Chirkov, 2002). Although the mechanisms of action are not completely understood, there are several hypothesized mechanisms related to chitosans positive charge below pH 6.3 (Liu et al., 2004). It is proposed that chitosan induces leakage of

intracellular material and alters transport of materials through electrostatic interactions with negatively charged components of the outer cell, controls growth through chelation of essential minerals, interferes with DNA transcription of microorganisms, or indirectly induce defense mechanisms of plants (Badawy and Rabea, 2009; Durango et al., 2006; El Ghaouth et al., 1992; Hadwiger and Loschke, 1981; Stossel and Leuba, 1986; Li et al., 2010; Rabea et al., 2003; Roller and Covill, 1999; Tharanathan and Kittur, 2003; Zheng and Zhu, 2003).

Foodborne viruses are recognized as one of the major causes of foodborne illness and death. Viruses are responsible for 5.5 of the 9.4 million illnesses (59%), 27% of the 55,961 hospitalizations, and 12% of the 1,351 deaths related to food contamination (Scallan et al., 2011). Of the viruses studied, the human norovirus was the leading cause for all three categories. Human noroviruses are enteric viruses that are considered to be the cause of more than 90% foodborne non-bacterial gastroenteritis or about 5.5 million reported cases annually (Mead et al., 1999; Patel et al., 2008; Scallan et al., 2011). However, this number may be underestimated due to milder cases not reported. As a member of the *Caliciviridae* family, human noroviruses have single stranded positive sense RNA genomes ~7.5kb in size (Jiang and Estes, 1990; Perry and Wobus, 2010) These viruses are non enveloped, have an isocahedral shape being about 27-38 nm in size, and only require about 10-100 particles to cause infection (Bok et al., 2009; Cheesbrough et al., 2000; Hyde et al., 2009; Taube et al., 2010). Human noroviruses have been shown to be sensitive to heat treatments and chlorine at concentrations >2mg/l, but have been shown to be less susceptible to pH (Cliver et al., 2002; Koopmans et al. 2002). . The major cause of contaminated food and water occurs through the fecal-oral route

through person to food contact, person to person contact, person to environmental surface, contaminated vomit, or aersolization (Greening, 2006; Patel et al., 2009; Sair et al., 2002). The major foods at risk are handled and ready to eat foods that do not undergo further cooking conditions, which include fresh produce, juices, shellfish, salads, and boxed lunches (Grove et al., 2006; Sair et al., 2002)

Since there is no lab host cell culture system for human norovirus propagation currently, cultivable surrogates, such as feline calicivirus (FCV-F9), murine norovirus (MNV-1), and coliphage MS2, are used in infectivity assays (Doultree et al., 1999; Duizer et al., 2004). As a member of the *Caliciviridae* family, FCV-F9 is used as a surrogate due to its genetic similarities (Bidawid et al., 2000; D'Souza et al., 2006). However, it differs from the human noroviruses because it is transmitted through the nasal, oral, or conjunctival routes causing a respiratory infection and is more susceptible to low pH than enteric viruses (Cannon et al., 2006; Duizer et al., 2004; Perry et al., 2009; Radford et al., 2007). As a member of the same *Calicivirdae* family and under the *Norovirus* genus, MNV-1is considered a better surrogate system to study the biology and pathogenesis of the human noroviruses by some researchers because it is more resistant to environmental conditions than FCV-F9 and is an enteric virus with similar clinical symptoms (Cannon et al., 2006; Green et al., 2001; Karst et al., 2003; Wobus et al., 2006). Bacteriophage MS2 is a member of the *Leviviridae* family and is commonly used as a surrogate for enteric RNA viruses in water contamination studies because it is adapted to the intestinal tract, (Dawson et al., 2005). It has similarities to human noroviruses, which include being positive sense, single stranded RNA virus about 22-29

nm in size with an isocahedral shape (Calender, 1988; Dawson et al., 2005; Guan et al., 2006; Langlet et al., 2007; Shin and Sobsey, 2003; Toropova et al., 2008). Phi X174, which is commonly used as an indicator for fecal contamination, was used a surrogate for single stranded DNA enteric viruses (Charles et al., 2009). As a member of the *Microviridae* family, phi X174 is a positive sense, circular, single-stranded DNA bacteriophage, about 30 nm in size with an isocahedral shape (Bennett et al., 2008; Bernhardt et al., 2002; Brentlinger et al., 2002; Ilag et al., 1994; Suzuki et al., 1999).

The objective of this study was to determine the effect chitosan concentration on the enteric virus surrogates FCV-F9, MNV-1, phi X174 and MS2.

2. Materials and Methods

2.1. Viruses, hosts, and cell lines

Coliphage phi X174 and its host *Escherichia coli* CN-13 (both received as a gift from Dr. Suresh Pillai of Texas A&M University, College Station, TX); Coliphage MS2 and host, *E. coli* B-15597 (both from ATCC, Manassas, VA); Feline Calicivirus F9 (FCV-F9) and cell line Crandell Reese Feline Kidney (CRFK) cells (both from ATCC, Manassas, VA); Murine Norovirus-1 (MNV-1; graciously provided by Dr. Skip Virgin, Washington University, St. Louis, MO) and host RAW 264.7 cells (from the collection of University of Tennessee at Knoxville) were used in this study.

2.2. Virus Propagation

For phi X174 and MS2, hosts *E. coli* CN-13 and *E. coli* B-15597 respectively, were transferred twice in 3% trypticase soy broth containing 0.1% glucose, 20µg/ml $CaCl_2$, and 10 µg/ml thiamine with an incubation period of 6 hr at 37°C. Following the second incubation, the viruses were added to their hosts for ~18 hr. The viruses were collected by centrifugation at 3,000 x g for 10 min and the supernatant was filtered through a 0.2 µm membrane filter. Lastly, 1 ml aliquots were placed into vials to be stored frozen at -80°C until use in the experiment. For MNV-1and FCV-F9, host cells Raw 264.7 and CRFK, respectively, were incubated at 37°C under 5% CO_2. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin-streptomycin and 10% heat inactivated fetal bovine serum (FBS). RAW 264.7 and CRFK cells were infected with MNV-1 and FCV-F9, respectively, and incubated at 37°C under 5% CO_2 until at least 90% lysis. The incubation time for MNV-1 was ~4-6 days and FCV-F9 was ~24 hr. After lyses, the viruses were harvested by freeze-thawing, three times for MNV-1 and once for FCV-F9. Next, the viruses were centrifuged at 5,000 x g for 10 min. Lastly, supernatants were filtered through a 0.2 µm membrane filters, 1 ml aliquots placed into vials, and frozen at -80°C until use in the experiment.

2.3. Chitosan application for viral inactivation

Water-soluble chitosan (MW 53 kDa, DA 9.1%; EZ Life Science Co. Ltd., Seoul, South Korea) dissolved in sterile deionized distilled water at a concentration of 1.4%, 2.0%, 2.5%, and 3.0% was mixed with equal volume of virus suspension in PBS to obtain

the final concentrations of ~5 $\log_{10}$ PFU/ml virus titer and 0.7%, 1%, 1.25%, and 1.5% chitosan and incubated for 3hrat 37°C. Chitosan of 222kDa and 32.5%DA (as determined in our lab, Primex, Iceland) was dissolved in 1% acetic acid and sterile deionized distilled water to form a chitosan concentration of 1.4%, 2.0%, 2.5%, and 3.0%. Chitosan solutions were mixed with equal volume virus suspension in PBS to reach a mixture with the final concentrations of ~5 $\log_{10}$ PFU/ml virus titer and of 0.7%, 1.0%, 1.25, and 1.5% chitosan in 0.5% acetic acid, and incubated for 3 hr at 37°C. Three controls were applied: (a) "3 hr" - a 3 hr incubation virus control (~5 $\log_{10}$ PFU/ml in PBS), (b) "AcAc4.5" - a virus control (~5 $\log_{10}$ PFU/ml in PBS) with acetic acid (~0.15%) to have pH similar to the pH of 222 kDa chitosan and virus mixture, (c) "AcAc5.6" - a virus control (~5 $\log_{10}$ PFU/ml in PBS) with acetic acid (~0.0625%) to have pH similar to the pH of 53 kDa chitosan and virus mixture. All treatments were done in duplicate and replicated at least twice.

2.4. Plaque Assay Infections

The technique of Bae and Schwab (2008) and Sue et al. (2009) was followed for MS2 and phi X174 plaque assays with exception that 0.7 ml of serially diluted treated and untreated bacteriophage MS2 was mixed with 0.30 ml of 5-6 hr *E. coli* B-15597 in 8 ml of 0.6 % top agar, and poured on tryptic soy agar (TSA) plates containing 0.5% NaCl. For phi X174, 0.7 ml of serially diluted treated and untreated phage phi X174 was mixed with 0.25 ml of 5-6 hr *E. coli* CN-13 host with 8 ml of 0.6 % top agar, and poured on

tryptic soy agar (TSA) plates. For both viruses, plates were incubated at 37^{o}C overnight before plaques were counted.

Plaque assays for MNV-1 were done similarly to Wobus et al. (2004) and Su et al. (2009). After confluent RAW 264.7 cells were grown in flasks, 2.0 ml of cells were added to each well in 6-well plates and incubated until ~90% confluent. Treated and untreated MNV-1 was serially diluted tenfold in DMEM-F12 containing 10% FBS. After the media of each 6-well plate was aspirated, it was inoculated with 0.50 ml of each virus dilution (treated and untreated controls) and incubated at 37°C under 5% CO_2 for 2.5 hrs. Again, the media of the 6-well plates was aspirated and the cells were overlaid with 2 ml of DMEM F-12 containing 0.75% agarose, 10% FBS and 1% penicillin-streptomycin. The 6-well plates were allowed to solidify and then incubated for 72 hrs before staining media, which contained the same ingredients as the first overlay media plus 0.02% neutral red, was added and allowed to solidify. Finally, the plates were incubated for 3-5 hrs at 37°C under 5% CO_2 before plaques were counted.

Plaque assays for FCV-F9 were done according to the procedure of D'Souza et al. (2006) and Su et al. (2009). CRFK cells were grown in flask at 37°C under 5% CO_2 until confluent. CRFK cells were added to 6-well plates (2 ml) and incubated until ~90% confluent. Treated and untreated FCV-F9 were serially diluted tenfold in DMEM F-12 containing 2% FBS. After the wells in the plate were aspirated, they were inoculated with 0.5 ml of the virus dilutions (treated and untreated controls) and incubated for 2.5 hrs at 37°C under 5% CO_2. The media of the 6-well plates were aspirated and then overlaid with DMEM F-12 containing 0.75% agarose, 2% FBS and 1% penicillin-streptomycin. The plates were incubated for 48 hrs and overlaid with another overlay media that

contained 0.01% neutral red along with the other ingredients of the previous overlay. The plates solidify before being incubated for no longer than 24 hrs under 5% CO2. Finally, the plaques were counted.

2.5. Statistical analysis

ANOVA and Tukey's test were determined on a completely randomized design with sampling using SAS software (version 9.2, SAS Institute, Cary, NC, USA) on the data from at least two replications with duplicates.

3. Results and Discussion

3.1 Effect of Chitosan on phi X174

Only concentrations of 1.25% and 1.5% of 53 kDa chitosan significantly reduced phi X174 compared to the 3 hr control, and only 1.5% 53 kDa was significantly different from the pH control (pH 5.6) by 0.47 log PFU/ml (Table 1). All other concentrations of 53 kDa and 222 kDa chitosan did not cause significant reduction of phi X174 (Table 1). Although the reduction was statistically significant ($P<0.05$) from the 3 hr control, the reduction of 1.5% 53 kDa chitosan was only 0.94 log PFU/ml. For phi X174, increasing the concentration shows little promise at reducing the recovery.

3.2 Effect of Chitosan on MS2

The greatest effect of chitosan was seen on MS2 compared to the other surrogates, which was the trend seen by increasing the MW of chitosan in Chapter III. All concentrations of 53kDa and 222 kDa chitosan caused significant reduction of MS2. Similar reductions of MS2 were found for both chitosan at 0.7% compared to our previous work (Chapter III) on the effect of chitosan molecular weight. The recovery of MS2 was not significantly affected by increasing the concentration of 53 kDa chitosan, but it was significantly affected by increasing the concentration of 222 kDa. Increasing the 222 kDa chitosan to 1.0% was sufficient to completely inactivate the virus. After taking into account the effect of acid on MS2, the four concentrations of 53 kDa and 222 kDa chitosan caused between a 2.0-2.3 and 1.6- 4.15 log PFU/ml reduction, respectively, (Figure 1, Table 1). Increasing the concentration of chitosan becomes effective against MS2 once a certain MW is reached.

3.3 Effect of Chitosan on FCV-F9

The FCV-F9 titer was significantlyreduced by all tested concentrations for both chitosans (53 and 222 kDa) compared to the controls. Increasing the concentration of 53 kDa from 0.7 to 1.5% did cause a significant increase in the reduction of FCV-F9 but it was only a 0.27 log PFU/ml difference (Table 1). Chitosan of 222 kDa in concentration of 0.7% reduced the titer for 2.22 PFU/ml compared to 0.95 PFU/ml in acidified control, but increase in concentration did not further reduce FCV-F9 titer (Figure 1, Table 1). Chitosan of 53kDa caused a more significant reduction of FCV-F9 than 222 kDa

chitosan, which was similar to the trend found in our previous work on the effect of molecular weight.

3.4 Effect of Chitosan on MNV-1

The recovery of MNV-1 was significantly reduced by all concentrations of both tested chitosans with exception of 1.25% 53 kDa (Figure 1). The titer reduction caused by 53 and 222 kDa chitosan treatments ranged between 0.31-0.35 and 0.82-0.95 log PFU/ml, respectively. Overall, increasing the chitosan concentration did not significantly affect the reduction of MNV-1 for either MW. Although the reduction obtained was greater using 222 kDa compared to 53 kDa chitosan, the maximum reduction was still below 1 log PFU/ml, and thus has little practical potential for application.

3.5 Discussion of chitosan concentration on antiviral effect

Bacteriophage MS2 showed the most vulnerability to chitosan. It showed similar susceptibility for both molecular weights at 0.7 % concentration compared to the previous study. Increasing the concentration only increased the antiviral activity for 222 kDa, which could be due to the large gap in MW between 53 kDa and 222 kDa. For phi X174, increasing the concentration of chitosan for both MWs was not effective at increasing the recovery of the virus. For FCV-F9, 53 kDa chitosan showed an increase in antiviral activity as the concentration reached a certain parentage (1.25%), but the antiviral activity for 222 kDa did not change with changes in concentration. MNV-1

showed significant reductions in titers due to 222 kDa at all concentrations and 53 kDa at all concentrations except 1.25%. Although, a clear trend of increasing antiviral activity with increasing concentration cannot be seen for all the four viruses tested, lower concentrations of chitosan do not appear to be more effective than the higher concentrations of chitosan on any of the tested viruses. The inactivation mechanism by chitosan needs to be examined to further explain the inactivation of viruses seen in this research.

The antiviral activity has been shown to be dependent on the concentration of chitosan in some earlier reports. Prospieszny et al. (1991) sprayed bean plants with chitosan concentrations ranging 0.00001-0.1% 15 min before inoculation with alfalfa mosaic virus and found increasing inhibition of the virus with increasing concentration where complete inhibition at 0.01% was obtained. Another study showed the increase in chitosan, chitosan acetate, and chitosan hydrochloride concentration from 0.00005 to 0.01% to cause an increase in infection inhibition from less than 50% to 100% against bacteriophage T2 and T7 (Kochkina and Chirkov, 2000). Still another study found conflicting results increased antiviral activity by increasing the concentration of chitosan Su et al. (2009) found an increase in concentration from 0.175, 0.35, to 0.7% to cause a statistically significant increase in the reduction of ~10^7 FCV-F9, which was 1.09, 2.09, 2.83 log PFU/ml, respectively, by 53 kDa chitosan and 0.44, 0.99, 1.44 log PFU/ml, respectively, by 5 kDa chitosan. A similar trend was seen by 53 kDa chitosan against ~10^5 FCV-F9 and 5 kDa chitosan against ~10^7 and ~10^5 FCV-F9 and ~10^5 MNV-1 (Su et al., 2009). Chitosan of 53kDa at 0.175% was shown to cause a statistically larger reduction (0.32 log PFU/ml) of ~10^5 MNV-1 compared to 0.7% chitosan of 53 kDa (0.04

log PFU/ml) (Su et al., 2009). Similarly, the same trend was seen for 53 kDa chitosan as concentration decreased against ~10^7 MS2 and MNV-1 and for 5 kDa chitosan against ~10^7 MS2. Some viruses were not affected by an increase or decrease in concentration. This was seen for 53 kDa chitosan against ~10^5 MS2 and for 5 kDa chitosan against ~10^5 MS2 and ~10^7 MNV-1 (Su et al., 2009). Still, more research needs to be done to determine effect of the concentration on foodborne antiviral activity.

The concentration of chitosan has been shown to influence the antibacterial activity against such organisms as *Staphylococus aureus*, *Escherichia coli*, *S.* Typhimurium and *Yersinia enterocolitica* (Helander et al., 2001; Liu et al., 2004; Wang et al. 1992). Kong et al. (2008) found the antibacterial activity of chitosan against *E. coli* to increase as the concentration of 1456 kDa chitosan was increased from 0.02 to 0.1%. Liu et al. (2004) and Zheng and Zhu (2003) found an increase in concentration ranging from <5 to 305 kDa to have increasing antibacterial action against of *E. coli* and *S. aureus* and ultimately cause inactivation. Also, chitosan was found to disrupt the outer membranes of *S.* Typhimurium at 0.01-0.025%, but did not kill it until reaching 2% (Helander et al., 2001). The increase in antibacterial activity by chitosan due to an increase in concentration has been found to occur at different rates depending on the type of bacteria, such as *Enterobacter aeromonas, E. coli*, *Bacillus cereus*, *Brochothrix thermosphacta*, *Lactobacillus sakei*, *L. plantarum*, *Photobacterium phosphoreum*, *Pseudomonas fluorescens, S.* Typhimurium, and *Y. enterocolitica* (Devlieghere et al., 2004; Wang, 1992).

Chitosan concentration also seems to play an important role in the effectiveness of antifungal activity. Increasing the concentration of chitosan concentration from 0 to 1.0%

has been shown to increase the antifungal activity of chitosan against *Botrytis cinerea Candida albicans, C. krusei, C. glabrata,* and *Penicillium expansium* (Liu et al., 2007; Seyfarth et al., 2008). One study found that as the concentration of chitosan increased from 2, 4, to 6% that the decay in strawberries was decreasing the decay by *B. cinerea* (Reddy et al., 2000). Badaway et al. (2009) found that *B. cinerea* treated for 3 days at 25˚C with the concentrations ranging 0.05 to 0.4% of molecular weight ranging between 5 and29 kDa, the antifungal activity increased causing complete inactivation at 0.2% for all molecular weights.

4. Conclusions

Increasing the concentration of chitosan from 0.7, 1.0, 1.25, to 1.5% was most effective for 222 kDa chitosan against MS2, followed by 53 kDa against FCV-F9, while ineffective for 53 kDa against MS2, for 222 kDa against FCV-F9, and for both chitosans against phi X174 and MNV-1. The recovery of MS2 was reduced by 222 kDa chitosan as the concentration of chitosan increased, with 1.0% being sufficient to completely reduce the virus titer. The recovery of FCV-F9 was reduced by 53 kDa chitosan as chitosan concentration increased to 1.25% or greater. Susceptibility of phi X174 and MNV-1 was not concentration dependent. Overall, increasing the concentration either increases the antiviral activity or does not change it. These results indicate that chitosan shows potential as an antiviral agent in the food industry as a packaging material, coatings, or sprays on crops, but the increasing the concentration above 0.7% does not appear to have a major effect on viral inactivation.

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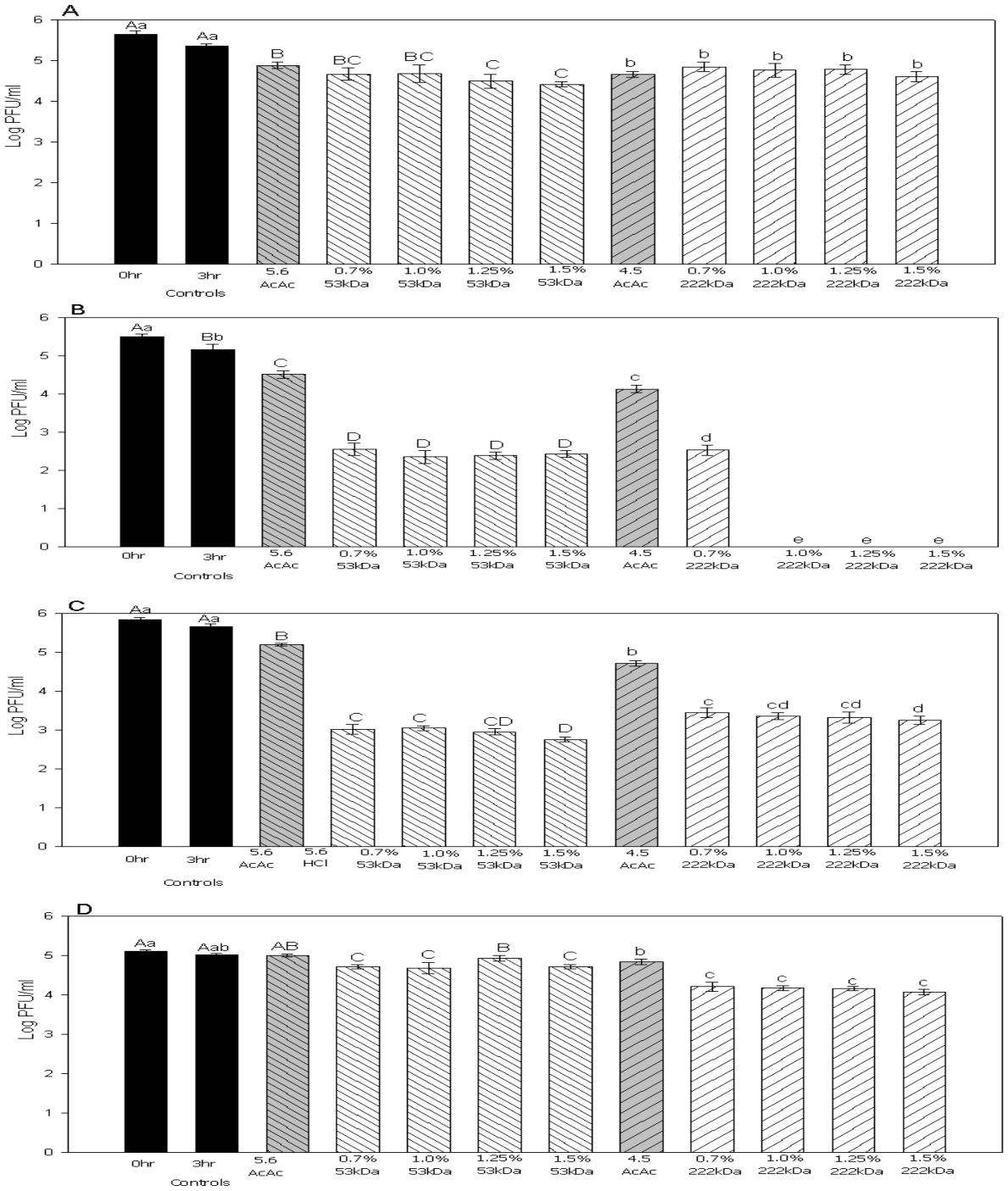
Appendix



1. Figure 1. Effect of 0.7, 1.0, 1.25, and 1.5% chitosan in water or acetic acid (AcAc) on the recovery of phi X174 (A), MS2 (B), FCV-F9 (C), and MNV-1 (D) using titers of ~5 log PFU/ml. (▨ 53 kDa at pH 5.6, ⫽ 222 kDa at pH 4.5). Dark colored is recovery of virus in PBS control after 0 and 3 hr. 53 kDa chitosan is water-soluble and therefore not carried out using HCl or AcAc that were done for the 222 kDa chitosan.

Table 1. Effect of pH controls with acetic (AcAc) and 0.7, 1.0, 1.25, and 1.5% of 53 and 222 kDa chitosan in water or acetic acid on the reduction of phi X174 phage, MS2 phage, FCV-F9, and MNV-1 using titers of ~5 log PFU/ml.

Treatment		Reduction (log PFU/ml)			
		Phi X174	MS2	FCV-F9	MNV-1
	3 hr control	$0.00^{Cb} \pm 0.00$	$0.00^{Cd} \pm 0.00$	$0.00^{Dc} \pm 0.00$	$0.00^{Bb} \pm 0.00$
5.6 pH	AcAc cont.	$0.47^{B} \pm 0.08$	$0.64^{B} \pm 0.09$	$0.47^{C} \pm 0.04$	$0.03^{B} \pm 0.04$
	0.7% 53 kDa	$0.69^{AB} \pm 0.15$	$2.61^{A} \pm 0.16$	$2.64^{B} \pm 0.02$	$0.31^{A} \pm 0.05$
	1.0% 53 kDa	$0.68^{AB} \pm 0.22$	$2.80^{A} \pm 0.17$	$2.62^{B} \pm 0.04$	$0.35^{A} \pm 0.14$
	1.25% 53 kDa	$0.86^{AB} \pm 0.17$	$2.77^{A} \pm 0.10$	$2.72^{AB} \pm 0.04$	$0.10^{B} \pm 0.07$
	1.5% 53 kDa	$0.94^{A} \pm 0.03$	$2.73^{A} \pm 0.09$	$2.91^{A} \pm 0.06$	$0.31^{A} \pm 0.05$
4.5 pH	AcAc cont.	$0.70^{a} \pm 0.07$	$1.02^{c} \pm 0.10$	$0.95^{b} \pm 0.08$	$0.19^{b} \pm 0.08$
	0.7% 222 kDa	$0.51^{a} \pm 0.12$	$2.63^{b} \pm 0.14$	$2.22^{a} \pm 0.09$	$0.82^{a} \pm 0.11$
	1.0% 222 kDa	$0.60^{a} \pm 0.17$	$5.16^{a} \pm 0.00$	$2.30^{a} \pm 0.04$	$0.86^{a} \pm 0.07$
	1.25% 222 kDa	$0.58^{a} \pm 0.12$	$5.16^{a} \pm 0.00$	$2.34^{a} \pm 0.09$	$0.87^{a} \pm 0.05$
	1.5% 222 kDa	$0.75^{a} \pm 0.13$	$5.16^{a} \pm 0.00$	$2.41^{a} \pm 0.04$	$0.95^{a} \pm 0.07$

* Reductions with similar lowercase and uppercase letters are statistically simililar within the entire column.

CHAPTER V
CONCLUSIONS AND RECOMMENDATIONS

In conclusion, chitosan was found to be efficient against MS2 and FCV-F9, while it had no effect on phi X174 and MNV-1. Reduction of MS2 by chitosan increased as molecular weight of chitosan increased, with high molecular chitosan (~1,150 kDa) being able to completely inactivate the virus from ~10^5 log PFU/ml. The inactivation of FCV-F9 and phi X174 were not molecular weight dependentwhile MNV-1, was not affected by chitosan. Overall, the molecular weight does play a role in the antiviral activity of chitosan against some of the tested viral surrogates.

Increasing the concentration of the chitosan showed varying affects on the four enteric virus surrogates. Increasing the concentration of chitosan from 0.7 to 1.5% was most effective for 222 kDa against MS2 and by 53 kDA against FCV-F9, while ineffective for 53 kDa against MS2, for 222 kDa against FCV-F9, and for both MWs against phi X174 and MNV-1. The infectivity of MS2 was completely inhibited by 1.0% or more 222 kDa chitosan. The reduction of FCV-F9 was significantly increased by 53 kDa chitosan as the chitosan concentration improved to 1.25% or greater. Susceptibility of phi X174 and MNV-1 was not concentration dependent.

The antiviral properties of chitosan depend on the type of virus and pH of the media. Overall, MS2 was most susceptible, followed by FCV-F9, phi X174, and MNV-1. The pH did have some effect on the recovery of all four surrogates. Lowering the pH from 5.6 to 4.5 caused a greater reduction in all viral titers. MS2 was affected the most by

the pH, followed by FCV-F9, phi X174, and MNV-1. However, comparing acetic and hydrochloric acid, the type of acid was not found to significantly affect the recovery of the any surrogates used in this research, except for MS2 and FCV-F9 with 222 kDa.

There is still need for research to determine the mechanism of antiviral activity of chitosan. One potential way to further the understanding of chitosan's antiviral activity would be to try the molecular weights (53 and 222 kDa) used in Chapter IV at lower concentrations to determine the effect on these surrogates. As well as 307, 421, and ~1,150 kDa chitosan could be tried at both lower and higher concentrations to determine their effect on these surrogates. Also, chitosan could be tried on different foodborne viruses. Further research could be done on the effect of degree acetylation of chitosan alone, degree acetylation combined with the concentration of chitosan, degree acetylation combined with molecular weight of chitosan, and all three taken into account. Future work should examine the interaction of chitosan and virus under the transmission electron microscope.

Chitosan does show potential for use in the food industry. Along with the antibacterial and antifungal properties of chitosan, it shows the potential to control the spread of some enteric viruses. As an antiviral, chitosan can be most effective at controlling the spread of viruses due to contamination from outside sources. As an antimicrobial chitosan can be applied as a package material or as a pre- or post harvest spray for crops that may become contaminated.

VITA

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